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ANTIMETABOLITES IN NUTRITION

By D. W. WOOLLEY, Ph.D.*

The past 15 years has seen the development of a new aspect of biochemistry which is making possible the understanding of several problems both of theoretical and practical importance in diverse fields of biological science. This aspect of biochemistry is the phenomenon of antimetabolites. The purposes of this review are to describe a few typical examples of the phenomenon, to outline the presently accepted concept of the mode of action of antimetabolites, and to illustrate a number of ways in which the phenomenon has advanced significantly the science of nutrition. Limitation of space necessitates the use of single examples to illustrate a fact where scores are known. The scope of the subject is evident when a recent monograph on antimetabolites was represented to be merely an abstract of existing knowledge, and yet it consisted of 269 pages (1).

Antimetabolites constitute a fitting subject for nutritionists because historically the field arose and was explored in the main by those studying the nutritional requirements of microorganisms and higher animals. True enough, the foundations of the concept were laid down by enzymologists, notably Michaelis (2, 3) and Quastel (4), but the flowering of the subject began, during a nutritional investigation, with the discovery of Woods (5) in 1940 that the bacteriostatic action of sulfanilamide and other sulfonamide drugs could be reversed in a competitive fashion by p-aminobenzoic acid. The growth-preventing action of the drug was overcome by a substance extracted from yeast or other organisms. When some of the chemical characteristics of this active substance became known, application of the ideas which had been developing in enzymology about enzymatic inhibition by structural analogs of the substrate led to the postulation that p-aminobenzoic acid, a close structural analog of sulfanilamide, was the active agent in the extracts. Experiments confirmed this postulation. Furthermore, the prediction was made that p-aminobenzoic acid would prove to be an essential growth factor, i.e. an essential metabolite, for several microorganisms, and this too was rapidly confirmed, not only by the finding of microorganisms which would not grow without it, but also by the discovery that it was a component of the folic acid (pteroylglutamic acid) of folic molecule. Thus, those species which did not show a nutritional need for this metabolite (p-aminobenzoic acid) still required it for vital processes, and consequently made it themselves. Nutritional need was an adequate but not a necessary criterion for a metabolite.

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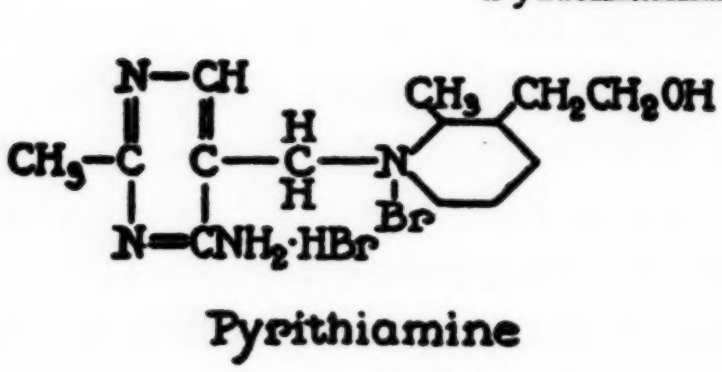
Production of Specific Vitamin Deficiency with Antimetabolites

The demonstrations with p-aminobenzoic acid vs. sulfanilamide could be made only in the nutrition of microorganisms, and depended on effects on the growth rate. In a short time, however, it was discovered that characteristic signs of vitamin deficiency diseases could be called forth in higher animals by the administration of a suitable structural analog of the vitamin in question. The syndrome so elicited could be prevented or cured by increasing the dietary intake of the particular vitamin. The signs called forth by the structural analog (which because it did call them forth we are entitled to name an antimetabolite) were produced while the animal was consuming an otherwise adequate diet. Thus, even though no deficiency was present in the ration, such a one was created in the animal by the antimetabolite. Since the signs resembled those which resulted from simple dietary restriction of the particular vitamin, and since they were obliterated by increases in the intake of this vitamin, it was clear that the antimetabolite was indeed creating a specific deficiency disease. In animals therefore, the case was clearer than in microorganisms where interpretation depended on growth rates. Except for vitamins A, D, and B_{12} antimetabolites of each of the vitamins have been produced and studied in experimental animals.

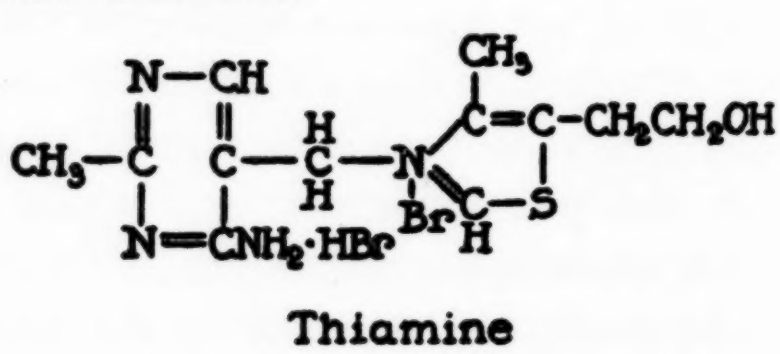
The first, and even today one of the clearest, examples is the production of of athiaminosis with pyrithiamine (6). (Sometimes known as neopyrithiamine (7)). The structure of this analog of thiamine is shown in Fig. 1 where its close resemblance to the vitamin can be seen. When pyrithiamine is fed to mice receiving an adequate, purified diet the typical manifestations of athiamin-

FIGURE 1

Pyrithiamine and Thiamine.



Pyrithiamine



Thiamine

osis are called forth (6). These can be either cured or prevented by administration of extra thiamine. The antagonism is competitive, so that if the dose of pyrithiamine is increased tenfold, the amount of thiamine needed to erase the disease is correspondingly increased.

The harmful effects of pyrithiamine, and the antagonism of these with thiamine, have been observed in several species of animals. They have also been studied in a wide variety of microorganisms (8). They have likewise

been seen in enzyme systems free of living cells. Thus, the synthesis of co-carboxylase from thiamine by an enzyme system in chicken blood was inhibited in a reversible fashion by pyrithiamine (9).

It is well known that the course of a specific avitaminosis can be profoundly influenced by more or less non-specific means. Thus, the acute crisis of black tongue in dogs, which is largely due to a deficiency of nicotinic acid, may be markedly alleviated by intravenous injection of physiological saline solution. The apparent recovery from the deficiency disease may last for weeks until the animal collapses from his primary avitaminosis. In view of such considerations it is indeed remarkable that so many of the signs of vitamin dificiency disease can be evoked with anti-metabolites. It would not be surprising if in some details the chemically induced syndrome should not correspond to the dietary insufficiency. In many of the conditions induced by the antimetabolites, as we see presently, some aspects of the disease are more prominent and others less so, than in conditions brought about by dietary restriction of the vitamin.

The fact that a given avitaminosis can be called forth by one antimetabolite in a variety of species illustrates the generality of the phenomenon. In the same way, the fact that such agents have been formed against practically all the vitamins makes it possible to investigate in this fashion a variety of avitaminoses. For these reasons the antimetabolites have been attractive substances to those who wish to study the clinical signs which may be produced in man by the lack of certain vitamins. As is well known, the carrying over to the practice of medicine of the knowledge of avitaminoses gained in animal experimentation is not easy. In the first place, it is difficult and sometimes impossible to maintain a group of men on a synthetic diet, and thus to produce a dietary lack of a certain vitamin. Even when such experiments can be arranged they usually involve the use of adults. Reflection on the results achieved in laboratory animals when adults rather than weanlings are employed, suggest that periods of depletion of many months, or even years, probably are required (10). This aggravates the difficulty of persuading men to submit to such experiments. There is also the problem of knowing when one has supplied all but one of the dietary essentials, since we do not know what the qualitative requirements of humans are. The few vitamins which are known with certainty to be required by man have been discovered by a discouragingly laborious and chance ridden process. For these reasons, and for others, the specific antimetabolites have seemed attractive tools with which to find the symptoms and signs of disease which might conceivably be attributed to the lack of some single dietary essential. However, because of the dangers inherent in the feeding of new and relatively unknown chemicals to human beings this aspect of the subject

is in its infancy. Without the use of antimetabolites we know that man needs a dietary source of vitamins A, D and K, and of thiamine, riboflavin, nicotinic acid, ascorbic acid, and, under conditions such as those obtaining in pernicious anemia, vitamin B_{12}. The status of other vitamins for man is still considerably in doubt. The kinds of signs one might expect to find in human folic acid deficiency can be glimpsed because a considerable body of information has been collected about the changes which occur after humans have received aminopterin. Aminopterin, the structural analog of folic acid, calls forth in a variety of animal and microbial species many of the changes which arise from dietary lack of this vitamin. It has also been given to humans as it has yielded some encouraging results in the management of leukemia. It is important to note that toxic manifestations of aminopterin can be counteracted effectively by the citrovorum factor, the metabolically active derivative of folic acid.

Another example of human experimentation of this sort has been the administration of the antipyridoxine, desoxypyridoxine, to human subjects (11). The results of this study may shed light on the kinds of disease one might expect to be associated with a lack of pyridoxine (vitamin B_6).

While studies of this kind illustrate what the signs of deficiency of a given metabolite may be in man, they do not tell us whether or not man has a dietary requirement for this metabolite. Just as with p-aminobenzoic acid in bacteria, the antimetabolites can show only that the metabolite to which they are related in chemical structure is vitally important. They cannot tell with certainty whether or not man can make his own vitamin or must rely on his food for it.

Mode of Action of Antimetabolites

From the beginning of the study of antimetabolites an understanding of the mode of action has frequently been found to be prerequisite to their effective use. This is not to say that the mode of action is known with certainty. What is known is a fruitful hypothesis which explains and accounts for most of the facts now at our disposal. This hypothesis envisions essential metabolites such as the vitamins, hormones, and amino acids functioning by acting as substrates in enzymic or semi-enzymic reactions. Thus, thiamine is known to act as substrate for the enzyme which synthesizes cocarboxylase (thiamine pyrophosphate). It is necessary to distinguish clearly here the fact that thiamine is the substrate in this reaction, and that cocarboxylase is the product. This is irrespective of the fact that in a separate, and unrelated enzyme system, cocarboxylase is a coenzyme for the decarboxylation of pyruvic acid.

The antimetabolite is pictured as exerting its effect because it, just like the metabolite, can combine with this specific enzyme. This union is accomplished because the antimetabolite, being a structural analog of the metabolite, has most of those chemical groupings which are responsible for the combination. This is why the antimetabolite must resemble the metabolite in chemical structure. However, the antimetabolite is sufficiently different from the metabolite that the enzyme finds it impossible to form the product from it. Consequently the enzyme is engaged with the foreign substance and cannot handle the normal metabolite to yield the usual product. The organism thus is deprived of this normal product. In the case of pyrithiamine it has been shown that this analog interferes with the synthesis of cocarboxylase, which is the normal product (9).

Some modification of this basic hypothesis has been found necessary in special cases. Thus, it seems that the hypoxanthine analog, aza-adenine, which is able to inhibit the growth of microorganisms, and to affect mice adversely (12) is also able to inhibit the utilization of hypoxanthine by xanthine oxidase (13). However, the enzyme does actually form products from the antimetabolite, but being thus engaged, it does not metabolize the normal substrate, viz. hypoxanthine. The essential point is still the same. The enzyme is prevented from carrying out its normal function, and the products of its normal action, which may be of vital importance, are not formed.

Antimetabolites in the Discovery of the Mechanism of Action of Vitamins and Other Metabolites

Because antimetabolites can be employed in normal organisms to block specifically the utilization of a given metabolite, they are proving to be powerful tools with which to discover the biochemical functions of the vitamins and other important compounds. Let us examine how this is being done by consideration of two examples: (a) the formation *in vivo* of purines and their derivatives in relation to folic acid, and (b) the discovery of a new metabolic function of thiamine.

When *Bacterium coli* was grown in the presence of sublethal amounts of sulfanilamide a diazotizable amine was found by Stetten and Fox (14) to accumulate in the medium. This compound was isolated in crystalline form. Shive and his collaborators (15) were able to identify it as 4-amino-5-carboxamidoimidazole. They were led to this finding in large measure from a consideration of the mechanism of action of antimetabolites. It was already known that the antibacterial effects of sulfanilamide could be overcome in a competitive fashion by p-aminobenzoic acid, and in a non-competitive way by purines such as xanthine, adenine or hypoxanthine, as

well as by substances such as methionine and folic acid. According to the postulated mechanism of action, the substrate (e.g. p-aminobenzoic acid) should interfere with the antimetabolite in a competitive fashion, whereas the product of the inhibited reaction should do so non-competitively. The purines and folic acid were non-competitive antagonists of sulfanilamide, and it was by then perfectly plain to see that folic acid was indeed a product derived from p-aminobenzoic acid by some series of reactions (16). Shive et al. postulated that the purines likewise were products arising from reactions in which p-aminobenzoic acid participated. When these reactions were blocked by the presence of the antimetabolite sulfanilamide, the precursors would normally be used to form the purines would go unused and would accumulate. 4-Amino-5-carboxamidoimidazole was a likely candidate for such a precursor, because it needed only the insertion of a single carbon atom to become hypoxanthine or xanthine. Shive et al. showed that the compound which accumulated in *B. coli* when inhibited with sulfanilamide was in fact this imidazole. Very recently, Greenberg (17) has demonstrated that it is the riboside of this base rather than the free imidazole which is formed. This is in harmony with the experiments done by classical enzymological techniques which had indicated that in the biosynthesis of purines, the nucleoside or nucleotide was formed first.

If p-aminobenzoic acid is a substrate from which organisms synthesize folic acid, and if, as Nimmo-Smith et al. (16) have shown, this synthesis is inhibited by the sulfonamide drugs, then it would probably be the lack of folic acid which was responsible for the failure of purine synthesis and the accumulation of 4-amino-5-carboxamidoimidazole or its riboside. Some evidence already existed to show that folic acid was in some way concerned with purine and thymine synthesis, because these substances (purines and thymine) could replace folic acid in the nutrition of *Streptococcus fecalis* (18). One could now predict that an antifolic acid would cause *B. coli* to accumulate the imidazole precursor of the purines just as sulfanilamide did. It was clearly a lack of folic acid which was the seat of the metabolic disturbance. The antifolic acid, aminopterin, was indeed found to cause the accumulation of the imidazole (19). Diverse nutritional and enzymological experiments have since made it clear that folic acid does function in the incorporation of single carbon atoms in several metabolic reactions, and, as we have seen, the imidazole differs from the purines by lack of one carbon atom.

There is another aspect of this involvement of folic acid with the formation of nucleic acids, which, because it was discovered through the use of antimetabolites, should be mentioned here. Shive et al. (20) inhibited the growth of *Leuconostoc mesenteroides* with the antifolic acid, x-methylfolic

acid, and observed that, in addition to folic acid, there was yet another substance in extracts of animal tissues which nullified the poisonous properties of the antimetabolite. They isolated this compound and identified it as thymidine, the desoxyriboside of thymine. From a consideration of the mechanism of action of antimetabolites, and of the behavior of thymidine vs. x-methylfolic acid they were able to conclude that folic acid was probably necessary for the formation of this desoxyriboside. Subsequently it has been learned that not only thymidine, but other desoxynucleosides as well are similarly concerned. The formation of desoxynucleosides, and therefore presumably of desoxynucleic acids, was thus indicated to depend on the functioning of folic acid. Simple dietary deficiency of this vitamin has now been shown to restrict the formation of these nucleic acids (21), at least in lactic acid bacteria.

The discovery of a new function for thiamine is an example which involves the extraordinary selectivity of antimetabolites in being able to discriminate between various metabolic functions of one vitamin. As we have seen above, pyrithiamine calls forth in animals the typical signs of thiamine deficiency, characterized by convulsions and retraction of the head over the back. Ever since the finding of thiamine as a part of cocarboxylase and the studies of Ochoa and Peters (22) on cocarboxylase and pyruvic acid levels in animals deprived of thiamine, the prevailing view has been that the neurological manifestations of athiaminosis arise from a lack of cocarboxylase with its attendant elevation of pyruvate. Certainly there can be no question but that cocarboxylase is much reduced in the tissues of animals deprived of thiamine, and that consequently blood pyruvate is increased. These changes were supposed to explain the neurological findings. The biochemical functioning of thiamine was thus to be understood in terms of cocarboxylase and of keto-acids. The observation that pyrithiamine would inhibit *in vitro* the synthesis of cocarboxylase by the enzyme system in chicken blood (9) thus provided a satisfying picture of the mode of action of this antimetabolite. However, the quantities required for this inhibition were large, and, in fact, much greater than those required to kill an animal. Examination of animals expiring from the effects of pyrithiamine showed that the cocarboxylase content of the livers was normal, and that the pyruvate level of the blood was not elevated. Clearly then, these animals had not died from an acute lack of cocarboxylase and the attendant hyperpyruvatemia (23). There must then be some new function of thiamine which is the one concerned with the neurological signs, and which is not involved with cocarboxylase directly.

In addition to pyrithiamine, Soodak and Cerecedo (24) have described another antimetabolite of thiamine. This is oxythiamine which differs from

the vitamin in having the amino group replaced by an hydroxyl. Oxythiamine calls forth in animals the retardation of growth and the anorexia associated with dietary deprivation of thiamine, and these signs can be overcome with this vitamin. However, oxythiamine does not evoke the neurological manifestations (25). The mode of action of this antimetabolite was studied by Frohman and Day (26) who found that when rats were given toxic amounts of it they excreted the thiamine which was displaced by the antimetabolite, and that the pyruvate level of their blood increased as it does in dietary athiaminosis. From such results one must conclude that oxythiamine functions primarily by interference with the synthesis of cocarboxylase, while pyrithiamine acts principally by inhibition of the postulated new function of thiamine, and only secondarily by retardation of cocarboxylase formation. Dietary lack of thiamine results in a failure of both processes.

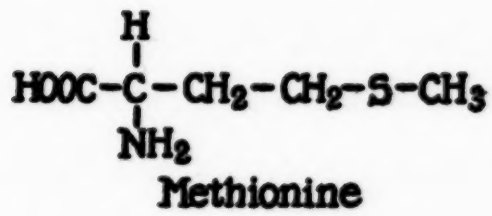
Antimetabolites in Foods and Elsewhere as Causative Agents of Disease

In years gone by it was standard practice to treat wheat flour with nitrogen trichloride in order to improve quality. This practice became suspect when it was discovered by Mellanby (27) that wheat treated in this fashion was responsible for running fits in dogs. The so-called canine hysteria, a disease seen in normal life, arose from the ingestion of foods containing such treated wheat products. The toxic substance in the flour was isolated in pure form by several laboratories (28–30) and identified as methionine sulfoximine. The structure of this substance is shown in Figure 2. It arises from the attack by nitrogen trichloride on methionine combined within the proteins of wheat. Curiously enough, free methionine does not yield the sulfoxamine when treated with nitrogen trichloride.

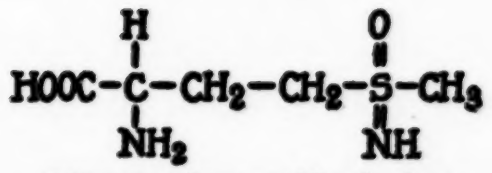
In tests conducted with rabbits (31) or lactic acid bacteria (32) the effects of methionine sulfoximine were found to be overcome by methionine, and thus it was an antimetabolite of this amino acid. Its convulsant action on dogs is presumed to be related to an antimethionine effect in the central nervous system. However, some experiments have been reported to indicate that more than this may be involved. Thus, Pace and McDermott (33) found that in rabbits that the glutamine content of the diet influenced the toxicity of methionine sulfoximine. In this connection it is of interest to recall that Borek and Waelsch (34) had demonstrated that, with lactic acid bacteria, methionine sulfoxide was an antimetabolite of glutamic acid and of glutamine. Methionine sulfoxide differs from the sulfoximine only in lacking the amino group on the sulfur atom. It may be that methionine and glutamine are metabolites functionally concerned with methionine sulfoximine.

FIGURE 2

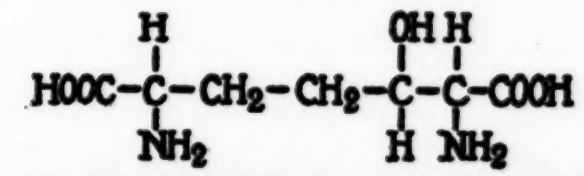
Methionine, methionine sulfoximine, and a new amino acid contained within the toxin of *P. tabaci*.



Methionine



Methionine sulfoximine

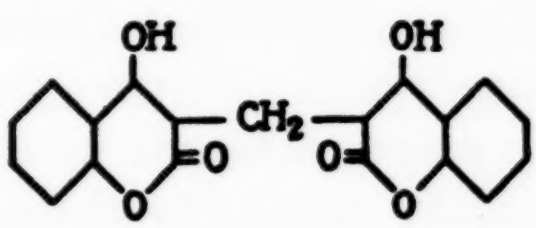


α,ε-diamino-β-hydroxypimelic acid
or
Tabtoxinine

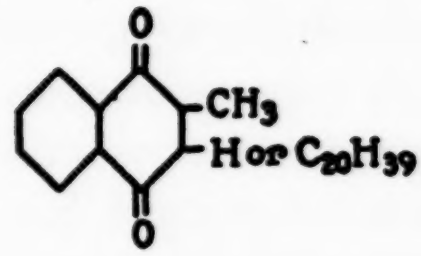
Methionine sulfoximine may be viewed as an antimetabolite unwittingly generated in food by the manufacturer. Although no ill effects have ever been noted in humans as the result of consuming flour bleached with nitrogen trichloride, the flour industry promptly changed their bleaching agent. There are reasons for suspecting that nature herself has put antimetabolites into materials used as food. Perhaps the best known example is that of dicoumarol, the causative factor of the hemorrhagic sweet clover disease. If sweet clover hay is cured or stored improperly it may develop the property of producing a hemorrhagic disease in farm animals which consume it. Insofar as is known, microbial action on the coumarin normally present in sweet clover leads to the formation of the poisonous substance. This poison was isolated and identified by Link and his collaborators (35) who found it to be of the structure shown in Fig. 3. Because the manifestations of the hemorr-

FIGURE 3

Dicoumarol (3,3′-methylenebis-4-hydroxycoumarin) and vitamin K.



3,3′-methylenebis-
4-hydroxycoumarin



Vitamin K

hagic disease induced by it were quite similar to the signs of vitamin K deficiency, attempts were made to prevent the disease with this vitamin. These proved successful. The structural resemblances of dicoumarol and vitamin K then began to seem important, capable of evoking specific deficiency diseases in animals. The view thus arose that dicoumarol was an antimetabolite of vitamin K, and owed its disease-producing powers in large measure to this.

The case for dicoumarol as an antivitamin K is not entirely convincing, however. In the first place, the resemblance in chemical structure is not as great as with, let us say, pyrithiamine and thiamine. In the second place, rather large amounts of vitamin K are needed to reverse the effects of one lethal dose of dicoumarol. If vitamin K dosage is increased tenfold, the amount of dicoumarol required to cause the hemorrhagic disease is increased, but not correspondingly. In other words, the antagonism is not competitive. These facts have tended to complicate the interpretation. Nevertheless, one should remember that the disease caused by dicoumarol has many of the specific signs of avitaminosis K, that the vitamin will prevent the disease, and that the poison somewhat resembles the vitamin in chemical structure. Add to this the fact that biochemical studies with radioactive dicoumarol have shown that the vitamin actually does displace the poison from combination in the liver (36). The displacement parallels the alleviation of the disease.

There is work to suggest that the time-honored association of corn-eating with the existence of pellagra may result from the presence of an antagonist of nicotinic acid in this food. From this grain a preparation has been extracted which depressed the rate of growth of mice maintained on a diet low in protein and in nicotinic acid. The deleterious effects of this concentrate were reversed by this vitamin or by tryptophan (37). The active principal was a basic substance which gave some indication of being a pyridine compound. However, it has not been characterized. The work of Aykroyd and Swaminathan (38) on the nicotinic acid content of corn-rich and corn-free human dietaries suggested that pellagra among corn-eaters could not be explained on the basis of insufficient intake of this vitamin. The later discovery (39) that tryptophan could serve as a source of nicotinic acid has shown that a low tryptophan content of food may accentuate the effects to be expected from a marginal level of the vitamin. At the present time most nutritionists attribute the pellagragenic action of corn to an amino acid imbalance coupled with a marginal content of nicotinic acid. Nevertheless, it may be well to remember that while zein, the principal protein in corn, is lacking in tryptophan, corn itself has an appreciable quantity of this amino acid. The fact that an antinicotinic acid can be extracted from corn without altering the amino acid content of the residual grain should make us reserve judgment on the final explanation of the pellagragenic action of this food.

Even if one admits the existence in corn of a pellagragenic agent the case is far from being clear. Until the substance is isolated and its chemical structure established it cannot be told whether or not it is an antimetabolite of nicotinic acid. It may well turn out to be unrelated in structure to the vitamin and to owe its effect to an entirely different mechanism. It may also prove to be active in mice but not in men.

Let us now examine a situation in which nature is seen to have elaborated an antimetabolite in one species, which while not being food for another, is pathogenic to this other organism. This is the case of the toxin of the pathogenic bacterium *Pseudomonas tabaci* which invades tobacco plants, causing necrotic spots to appear on the leaves. These lesions are caused by an extracellular toxin produced by the parasite. This toxin is deleterious to other species of plants in addition to the tobacco, and can, for example, be shown to inhibit the growth of an alga such as *Chlorella vulgaris.* In this alga the effects of the toxin can be overcome, and in a competitive fashion, by methionine, and by no other known substance (40). The postulate naturally arose that this toxin was nothing but a structural analog of methionine. The toxin has recently been isolated, and its structure partially elucidated (41, 42). It proved to be an amino acid. Hydrolysis of it yielded an acyl group and the compound shown in Fig. 2. The relationship to methionine can also be seen in this figure. Methionine sulfoximine, which stands as an intermediate between the two, was found to reproduce in tobacco leaves the lesions caused by the toxin.

In conclusion, it should be noted that physiological roles in normal metabolism are apparently played by pairs of structural analogs found side by side in the same individual. Thus, the antagonistic hormones testosterone and estrone, or progesterone and estrone, may be viewed as antimetabolites of each other.

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